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# Photodissociation of Human Oxy-Hemoglobin

## Studied by Time-resolved Photoacoustic Calorimetry<sup>1</sup>

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<sup>1</sup> Paper presented at the Fifteenth Symposium on Thermophysical Properties, June 22-27, 2003, Boulder, Colorado, U.S.A.

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### ABSTRACT

Time-resolved photoacoustic calorimetry is used to study the dynamics in nanosecond time scale for the photodissociation of human oxyhemoglobin. The enthalpy change  $H_R$  about 37.8 kcal/mol and the volume change  $V_R$  about 5.0 mL/mol due to the ligand dissociation are obtained and illustrated. Some influencing factors on experimental results are also discussed.

**KEY WORDS:** enthalpy change; hemoglobin; time-resolved photoacoustic calorimetry; volume change.

### 1. INTRODUCTION

Hemoglobin has always been the most extensively studied allosteric protein, which consists of four subunits, two denoted  $\alpha$  and two denoted  $\beta$ . The physiological role of hemoglobin is to bind oxygen molecules in lungs and carry them to tissues throughout the body, where these oxygen molecules are released. The efficiency of this role stems from the cooperative nature of the ligand binding in hemoglobin. Although there is a good description of the initial and final states of ligand binding[1-3], little is known regarding intermediate states that play a dominate role in cooperative ligand binding. However, with the development of time-resolved photoacoustic calorimetry (PAC)[4-6], especially in the last decade, it is now feasible to measure the dynamics of both volume and enthalpy changes accompanied with the photoinduced ligand dissociation on the time scale from picoseconds to microseconds. In early studies of Peters et al.[7], they successfully employed PAC method to understand the association and dissociation reactions of ligand of hemoglobin, in which they transiently created deoxyhemoglobin through ligand photodissociation of carboxyhemoglobin (HbCO). The choice of CO as a ligand is due to the fact that the quantum yield for ligand photodissociation of HbCO is

much greater than that of oxyhemoglobin ( $\text{HbO}_2$ ). In this paper, applying PAC method to study the human  $\text{HbO}_2$ , the results of the enthalpy and volume changes for ligand dissociation of  $\text{HbO}_2$  have been obtained. Some influencing factors on experimental results are also discussed.

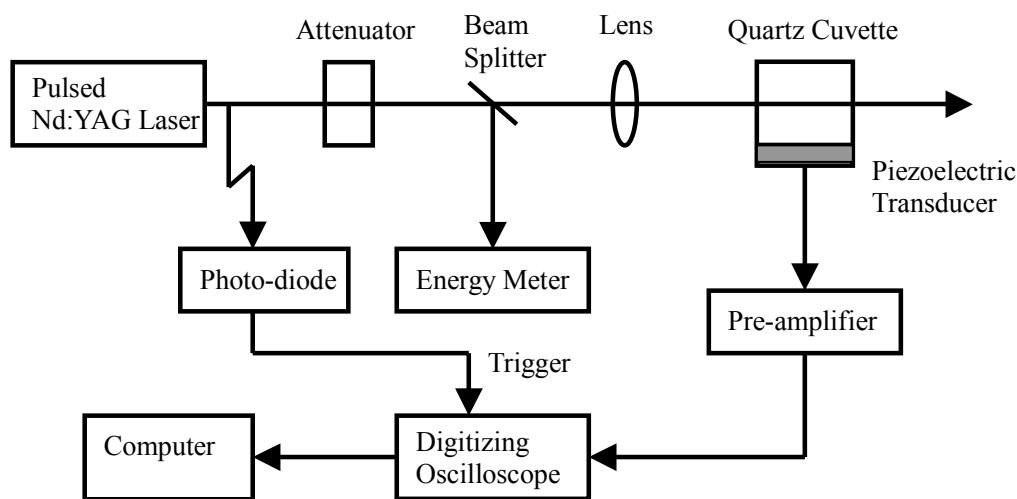
## 2. MATERIALS AND METHODS

### 2.1. Materials

Human hemoglobin solutions are prepared according to previous published procedure<sup>8</sup>, which are firstly dialyzed with 50mM phosphate buffer at pH 7.0 and then reduced with sodium dithionate ( $\text{Na}_2\text{S}_2\text{O}_3$ ). Then  $\text{HbO}_2$  is formed by bubbling  $\text{O}_2$  through the solutions. Meanwhile, the bromocresol purple (BCP) solutions in the same buffer as that for  $\text{HbO}_2$  are also prepared as a calorimetric reference. The absorbance of the solutions for both sample and reference at the excitation wavelength is determined using UV-3100 Spectrophotometer before the experiments.

### 2.2. Apparatus

The experimental schematic diagram for time-resolved PAC measurements is shown in Fig 1.



**Fig .1.** Experimental Setup of Photoacoustic Calorimetry

The excitation source is provided by a focused pulsed laser beam generated from a Nd:YAG laser that is operating at 532nm. The width of the laser pulse is 8ns and the repetition rate is 10Hz. A small part of scattering laser is detected by a photoelectric diode to trigger a digital oscilloscope. The laser intensity can be attenuated by a circular variable neutral density filter to the desired intensity, namely about 8~20  $\mu\text{J}$  per pulse. The photoacoustic signals are detected by a PZT transducer with a resonant frequency of 1.5MHz. The output of the transducer is amplified by a preamplifier and then

time-resolved to display by an oscilloscope with the maximum sampling rate of 5GHz. As noted in Fig 1., the laser energy is monitored and measured by an energy meter so as to complete the data analysis after the experiments. A 1-cm<sup>3</sup> right angle quartz cuvette is used to contain the solution which is kept slightly flowing with the speed about 1mL/min during the experiments. The temperature of solutions is varied from 7°C to 22°C with an interval about 2°C, which is controlled by a thermostat and measured with a thermal resistor in the cell. The absorbance of the aqueous solution of the sample is in the range of 0.15 ~ 0.25 at the optical wavelength of 532nm and is matched to the reference of the calorimetry.

### 2.3. Principle of method

The photoacoustic signal  $S$  is produced by the optical absorption of the irradiated sample. There are two main contributions to the detected acoustic signals. One is the thermally induced volume change in the solution,  $V_{th}$ , which is related to the thermal expansion coefficient  $\beta$  and the heat capacity  $C_p$  of the solution. The other is the molecular volume change  $V_{con}$ , which is due to the conformational change of the solution. Both contributions to detected signals can be expressed as[6]

$$S = K ( V_{th} + V_{con} ) \quad (1)$$

$$V_{th} = (\beta/C_p\rho) H$$

where  $K$  is the function of instrument response,  $\rho$  is the density and  $H$  is the released thermal energy during the photolysis process of the solution. In our experiments, in order to eliminate the function  $K$ , a calorimetric reference, bromocresol purple (BCP) solution, is used, which is known to convert the entire absorbed photon energy  $E_{hv}$  into heat with no reaction volume change with a time scale much faster than the time resolution of the experimental instrument. Thus the photoacoustic signals for the calorimetric reference  $S_{cal}$  is

$$S_{cal} = K (\beta/C_p\rho) E_{hv} \quad (2)$$

Dividing the sample's signal by that of the reference calibration gives the amplitude ratio  $\varphi$  is given as

$$\varphi = S / S_{cal} \quad (3)$$

Rearranging gives

$$E_{hv}(\varphi-1) = - H + V_{con}/(\beta/C_p\rho) \quad (4)$$

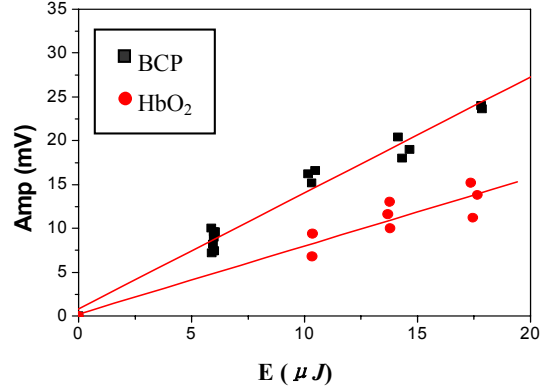
Here we define  $E_{hv}-H$  as  $H$ , the enthalpy change for the intermediate formation of HbO<sub>2</sub>. As the term  $\beta/C_p\rho$  is greatly changed with temperature in aqueous system, measuring  $\varphi$  as a function of temperature allows for the correlation of  $E_{hv}(\varphi-1)$  with  $\beta/C_p\rho$  yielding as the slope  $V_{con}$  and the intercept  $H$ . However, the quantum yield  $Q$  of the photo-induced chemical reaction must be taken into account for the evaluation of  $H$  and  $V_{con}$ . Therefore, the overall enthalpy change  $H_R$  and volume change  $V_R$  can be obtained as

$$H_R = H / Q \quad (5)$$

$$V_R = V_{con} / Q \quad (6)$$

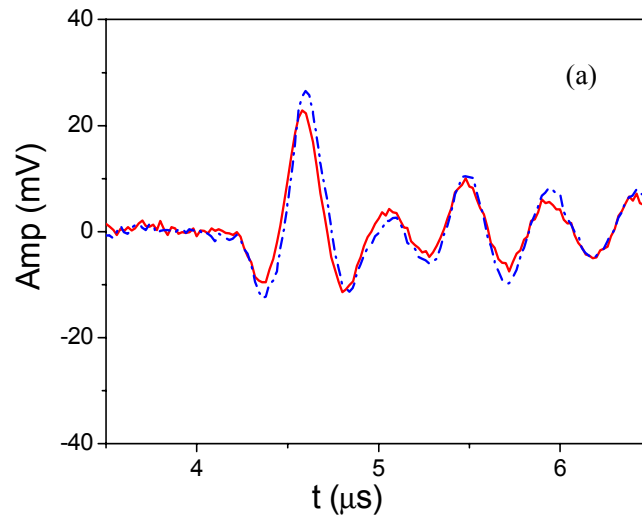
### 3. EXPERIMENTAL RESULTS

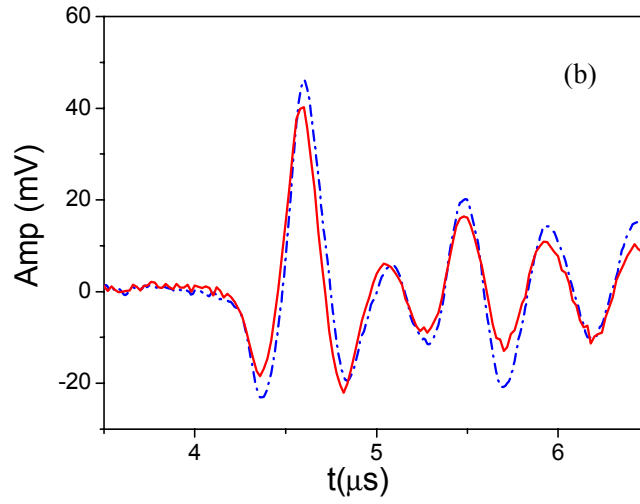
Firstly, the photoacoustic signals of both HbO<sub>2</sub> and BCP over the optical pulse energy range 0~20  $\mu$ J are measured to make sure the linearity of the photoacoustic response with pulse energy as shown in Fig.2 (taking the signals at 18°C for example), which avoids the possible multi-photon effects in solutions. During the experiments, a waveform baseline is collected for 100 laser pulses with no laser light incident on the cuvette. By carrying out photoacoustic experiments at different temperatures ranging from 7 °C to 22 °C, photoacoustic signals of both HbO<sub>2</sub> and BCP in 50mM phosphate buffer, pH7.0 are obtained, and the signals at 13.3 °C and 20.2 °C are shown as examples in Fig. 3(a) and 3(b) respectively. Each of the photoacoustic waveforms must be normalized, including subtracted by the waveform baseline and divided by the solution absorbance factor ( $1-10^{-A}$ , where A is the absorbance at the excitation wavelength, 532nm) and the average laser pulse energy  $E_0$ . After that, the value of S (HbO<sub>2</sub>)/S (BCP) at the temperature ranging from 7 °C to 22 °C can be obtained.



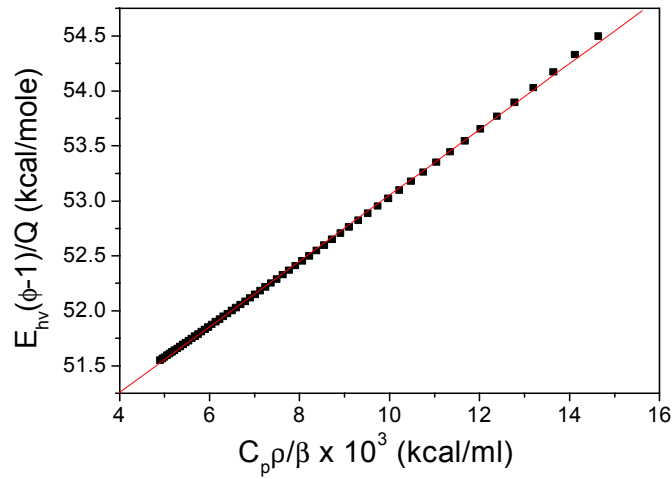
**Fig.2.** Photoacoustic signals vs.pulse energy

One important factor that deeply influences the results is how the value of  $\beta/C_p\rho$  changes with temperature. In our experiments, we compare the PA signal amplitudes of





**Fig.3.** Photoacoustic Signals of HbO<sub>2</sub> (solid line) and BCP (dash dot line) at (a) 13.3 °C and (b) 20.2 °C



**Fig. 4.**  $E_{hv}(\phi-1)/Q$  vs.  $C_p \rho / \beta$  (HbO<sub>2</sub> in 50mM phosphate buffer at pH7.0)

the BCP in different buffered solutions as a function of temperature by the same method as the prior studies of Peters et al.[7]. Furthermore, for another essential factor  $Q$ , the quantum yield for photodissociation of human HbO<sub>2</sub>, the value could be obtained with the similar methods in the published papers [7,9], which is 0.15.

From our experiments, we get a series values of the correlation of  $E_{hv}(\phi-1)/Q$  with  $C_p \rho / \beta$ , and one of them is shown in Fig.4, from which we could get the values for human HbO<sub>2</sub> with  $H_R = 37.8 \pm 3$  kcal/mol and  $V_R = 5.0 \pm 1$  mL/mol.

#### 4. DISCUSSION

The interpretations of the events after photolysis of oxyhemoglobin are generally

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with a hybrid of two models. One is the two-state model, the quaternary states of Hb comprise a high-affinity or relax state (R) and a low-affinity or tense state (T)[10]. The other is the sequential model, that is, the association and disassociation of ligands are a sequence of four distinct equilibrium steps corresponding to the successive binding of ligands to deoxyHb and the three partially ligated Hb species [11]. Considering these models, Perutz [12] presented a theory of three distinct changes involving in the process from the bound state to the ligand-free state HbO<sub>2</sub>, which are the movement of the iron atom relative to the heme plane, the tertiary conformation change caused by this movement and the quaternary conformational change following the tertiary change. In addition, the dynamics of O<sub>2</sub> dissociation from HbO<sub>2</sub> have been clearly studied with time-resolved descriptions on picosecond, nanosecond and microsecond time scales. Picosecond photolysis experiments for HbO<sub>2</sub> carried by Chernoff et al. [13] reveal the first geminate recombination process with half-times of 200ps. Based on the nanosecond time-resolved absorption study of photolysis intermediates of HbO<sub>2</sub>, Ebrahim et al. [14] demonstrated the slow geminate recombination process with a lifetime of 38ns and the tertiary relaxation with a lifetime of 137ns during the photolysis of HbO<sub>2</sub>. Following those processes, there are two microsecond ligand recombinations of O<sub>2</sub> to  $\beta$  and  $\alpha$  chains of Hb with lifetime values of about 11 $\mu$ s and 31 $\mu$ s respectively [15].

Considering the effective time scale of 100ns ~10 $\mu$ s which mainly determined by the pulse width of the exciting laser (8ns) and the bandwidth of the transducer (1.5MHz) used in our experiments, the reported geminate recombination processes of 200ps and 38ns are both too fast to be observed. Thus the changes we detected may be due to the 137ns tertiary relaxation. Then the volume change associated with this formation corresponds to the tertiary change in the R quaternary state without any quaternary structure changes, as described by both Perutz [12] and Ebrahim et al.[14]. Our observation is also in line with the report that for HbO<sub>2</sub> under conditions of low photolysis at 1 atm oxygen pressures, the quaternary transition is widely separated or lagged in time from the time scales required for the completion of the rebinding between O<sub>2</sub> and hemoglobin with the lifetime of microseconds [16].

With a developed thin-layer microcalorimeter method, Peters et al. [17] studied the stepwise enthalpies associated with the binding of four O<sub>2</sub> to deoxyhemoglobin in 0.1 M phosphate buffer at pH 7.6, as shown in Table I, from which we can get for human HbO<sub>2</sub>, the total enthalpy change of 48.1 $\pm$ 10 kcal/mol and the average enthalpy change per heme of 16.0 kcal/mol. Compared with the enthalpy change values we obtained about 37.8 kcal/mol, which are smaller than the total enthalpy of four O<sub>2</sub> to deoxyhemoglobin while greater than the average value per heme, it is reasonable to conclude that the transient photoproducts after the photolysis of HbO<sub>2</sub> in our experiments are mixtures of all partially ligated species R<sub>0</sub>, R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub>, which correspond to the non-ligated, single-ligated, dual-ligated and triplex-ligated species in R state respectively. Furthermore, because of the low photolysis yield employed in our

study, the process we observed during experiments is predominantly the relaxation in and ligand recombination to the triply ligated species  $R_3$ . It is hard to observe any transition between the two quaternary structures, R and T, because  $R_0$  and  $R_1$  are produced only in small amounts, their rapid transformations to the respective T species must be masked by other processes. Thus the partially ligated photoproducts in our experiments can be mainly the triply ligated species.

**Table I.** Intrinsic Human Hemoglobin Stepwise Thermodynamics (at 25 °C)

Process	$\text{Hb} + \text{O}_2 \leftrightarrow \text{HbO}_2$	$\text{HbO}_2 + \text{O}_2 \leftrightarrow \text{Hb}(\text{O}_2)_2$	$\text{Hb}(\text{O}_2)_2 + \text{O}_2 \leftrightarrow \text{Hb}(\text{O}_2)_3$	$\text{Hb}(\text{O}_2)_3 + \text{O}_2 \leftrightarrow \text{Hb}(\text{O}_2)_4$
H(kcal/mol)	$-10.3 \pm 1.1$	$-16.8 \pm 2.0$	$-0.7 \pm 4.6$	$-20.3 \pm 4.3$

## ACKNOWLEDGMENTS

This work is supported by the National Natural Science Foundation of China, No.10174038.

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